Enantioselective analysis of amino acids by capillary electrophoresis with fluorescence and mass spectrometric detection

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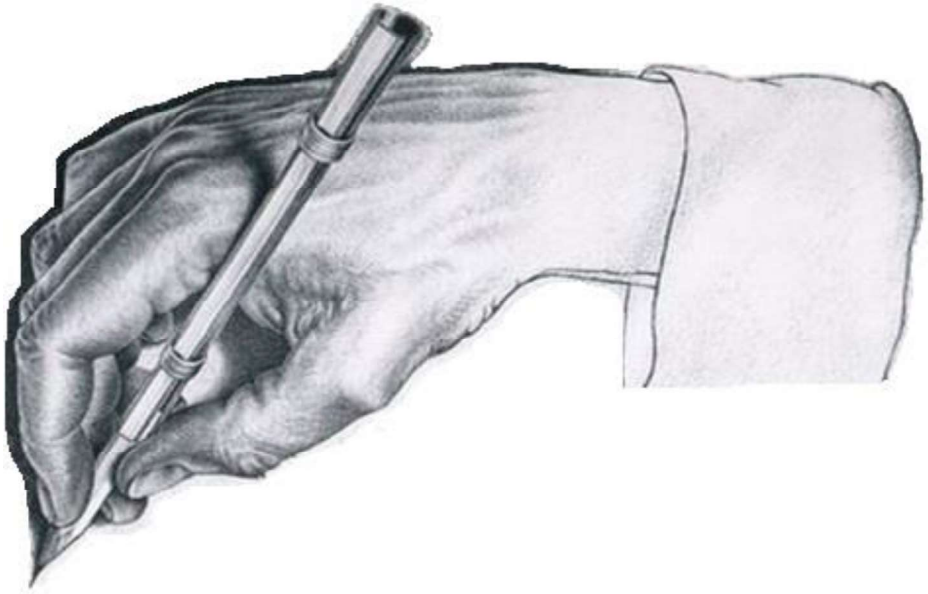
Chapter 6



Summary and concluding remarks

6.1. Summary

In this thesis, the potential of CE for the chiral analysis of proteinogenic AAs in biofluids was studied. For this purpose, chiral CE methods were combined with fluorescence (Flu) spectroscopy and mass spectrometry (MS) in order to gain detection sensitivity and selectivity. The CE separation was aimed to provide not only enantioresolution (i.e. separating DL-AA enantiomers), but also chemoresolution (i.e. separating AAs from each other and from matrix components). Overall, two general strategies for achieving enantioseparation of AAs were pursued: a direct and an indirect approach. In the direct method, a cyclodextrin (CD) was added as chiral selector to the background electrolyte (BGE) in order to attain separation of the AA enantiomers. In the indirect approach, the AA enantiomers were first derivatized with a pure chiral reagent to form diastereomers, which subsequently were separated using micellar electrokinetic chromatography (MEKC). For each approach, methods employing both Flu and MS detection were developed. The applicability of three of the newly developed methods was investigated by the chiral analysis of AAs in cerebrospinal fluid (CSF), evaluating the possibility to detect endogenous D-AAs.

Chapter 1 of this thesis provided an introduction to the research topic and described the scope of the thesis. In **Chapters 2 and 3** a direct approach for separating AA enantiomers was followed, employing β-CD as chiral selector in the BGE.

In **Chapter 2** the fast derivatization of AAs with the reagent FMOC is employed, allowing the use of UV-excited Flu detection for the enhanced detectability of the separated AA enantiomers. As D-AAs often are very minor components in complex multi-component samples, high selectivity and sensitivity is needed for their analysis and common UV-absorbance detection, therefore, may not be suitable. Only few aromatic AAs show native UV-absorbance and fluorescence. Optical detection of D-AAs can be achieved by their derivatization with UV or visible light absorbing reagents. Further increase of sensitivity is possible when fluorescent labels are used. Derivatization agents will not only improve the DL-AAs detectability, but can also aid the detection selectivity as only specific components in a sample will be derivatized and consequently detected. In this study, a Xe-Hg lamp was used as light source, allowing excitation of the FMOC-derivatized AAs in the UV range. A dedicated Flu detection cell that employs a ball-lens to focus the excitation light onto the capillary detection window was used. A dedicated optical cone employing wave-guiding principles permitted efficient emission light collection while rejecting reflected excitation light. The emitted light was guided to a spectrograph equipped with a charge-coupled device (CCD). Additional sensitivity enhancement was demonstrated

to be possible by signal averaging over time and emission-wavelength intervals, significantly improving the signal-to-noise ratio.

The chemo- and enantioseparation of FMOC-AAs was optimized by varying the concentration of the employed CD (chiral selector), the isopropanol content of the BGE, and the capillary temperature. The use of SDS as surfactant in the BGE, establishing MEKC system, showed good potential for enhanced chemo- and enantioseparation. A BGE of 40 mM sodium tetraborate (pH 9.5), containing 15% isopropanol (v/v), 30 mM SDS and 30 mM β-CD was found optimal for the chemo- and enantioseparation of FMOC-AA enantiomers. Enantioresolutions of 1.2 or higher were achieved for 15 DL-AAs. Overall performance was demonstrated by analysis of a mixture of 11 DL-AAs, showing their simultaneous chemo- and enantioresolution. Detection limits ranged from 14 to 98 nM injected concentration (which corresponds to 280-1960 nM in the sample before derivatization) for most AA enantiomers, and linearity (R^2 > 0.997) and peak-height (RSDs < 7%) and electrophoretic mobility (RDS < 0.6%) repeatability were satisfactory. The applicability of the optimized method was studied by the analysis of CSF. Endogenous levels of D-glutamine (565 nM) and D-aspartic acid (1365 nM) could be measured in CSF next to abundant L-AAs revealing enantiomeric ratios of 0.35% and 19.6%, respectively.

In **Chapter 3**, MS was coupled to chiral CE using the direct approach for DL-AA separation in order to achieve unambiguous assignment of separated AA enantiomers in complex, multi-component samples. β-CD was used as chiral selector and ESI-MS of the CE effluent was carried out via sheath liquid (SL) interfacing. Prior to analysis, AAs were fastly derivatized with FMOC, which showed to improve both their enantioseparation and ESI efficiency. The type of chiral selector type and its concentration in the BGE were studied regarding attainable chemo- and enantioseparation of DL-AAs and its effect on their MS detection. In addition, the effect of the pH of the BGE and its isopropanol content, the composition of the SL, and MS-interfacing parameters were evaluated. The optimized BGE consisted of 10 mM β-CD in 50 mM ammonium bicarbonate (pH 8.0) containing 15% isopropanol (v/v). The applied SL was isopropanol-water-1 M ammonium bicarbonate (50:50:1, v/v/v). Using these conditions, 15 proteinogenic AAs could be separated and detected exhibiting enantioresolutions of up to 3.5 and detection limits of 0.9-12.5 µM injected concentration (which corresponds to 3.6-50 µM before derivatization). The chiral CE-MS method was found suitable for the specific and selective detection of D-AAs spiked in CSF. Repeatability and linearity obtained for CSF were satisfactory, with peak area and migration-time RSDs below 17% and 2%, respectively, and a linear response (R^2 > 0.997) up to 100 µM injected concentration. The CSF results were similar to the

results obtained for aqueous solution of the same AAs enantiomers, indicating matrix effects were limited. Compared to previously reported works [1-3], the presented method allowed enantioresolution of a larger number of proteinogenic DL-AAs in one run and much shorter derivatization times (10 min vs. up to 16 hours).

In **Chapters 4 and 5**, chiral resolution of AA was achieved by first transforming the AA enantiomers to diastereomers using the chiral derivatization agent FLEC, and then separate these derivatives using MEKC, i.e., without the need of chiral selector in the BGE.

In **Chapter 4**, lamp-based Flu detection is used for the selective analysis of (+)-FLEC-derivatized proteinogenic AAs. As the diastereomers formed from the D and L enantiomer of one AA have identical charge and mass, their CE resolution required the presence of SDS above the CMC in the BGE (i.e. performing MEKC). The effects of the pH and composition of the BGE (SDS concentration, organic solvent type and percentage), and the capillary temperature and injection volume on the chemo- and enantioseparation were evaluated. A BGE of 40 mM sodium tetraborate (pH 9.2), containing 15% isopropanol (v/v) and 25 mM SDS allowed enantioseparation of twelve DL-AAs with chiral resolutions ranging from 1.2 to 7.9. UV excitation of the FLEC-AAs was achieved using the Flu detection setup described in Chapter 2. For the final emission detection, a comparison between a photomultiplier (PMT) and a spectrograph equipped with a CCD camera was made, showing that the ultimately achievable signal-to-noise ratios (S/N) were 23-times higher using the CCD detector. LODs for most AAs were in the 13-60 nM range injected concentration (which corresponds to 130-600 nM before derivatization). This encompassed an up to 100-times sensitivity improvement as compared to UV absorbance detection. For the chiral proteinogenic AAs, concentration linearity (R^2 > 0.985) and peak-area and migration-time repeatabilities (RSDs lower than 2.6% and 1.9%, respectively) were satisfactory.

Chapter 5 presents a new enantioselective CE-MS method in which the use of a chiral selector is circumvented by employing FLEC as chiral AA derivatizing agent and ammonium perfluorooctanoate (APFO) as a volatile pseudostationary phase for separation of the formed diastereomers. The DL-AAs were efficiently derivatized by a simple and fast mixture reaction with (+)-FLEC. CE-MS compatibility is greatly enhanced, as the separation of the formed FLEC-DL-AA diastereomers does not require a chiral selector in the BGE, avoiding related contamination of the ion source and suppression of the ESI of the analytes. In order to achieve diastereomer separation under MS compatible conditions, SDS was replaced by APFO as a negatively-charged volatile surfactant, capable of forming micelles and performing

MEKC separations. The effect of APFO on the MS signal of FLEC-AAs was studied, showing that APFO caused even less ion suppression than equal concentrations of ammonium acetate. In order to achieve optimized enantioseparation, the effect of the pH and APFO concentration of the BGE and the capillary temperature were studied. Furthermore, optimization of CE-MS parameters, such as SL composition and flow rate, ESI and MS settings was performed in order to prevent analyte fragmentation and achieve sensitive detection. Selective detection and quantitation of 14 chiral proteinogenic AAs was achieved with chiral resolution between 1.2 and 8.6, and LODs ranging from 130 to 630 nM injected concentration (which corresponds to 520-2520 nM in the sample before derivatization). Aspartic acid and glutamic acid were detected, but not enantioseparated. The optimized method was applied to the analysis of chiral AAs in CSF. Good linearity ($R^2 > 0.99$) and acceptable peak area and electrophoretic mobility repeatabilities (RSDs below 21% and 2.4%, respectively) were achieved for the chiral proteinogenic AAs, with sensitivity and chiral resolution mostly similar to obtained for standard solutions. Analyzing CSF from three healthy volunteers, endogenous levels of D-serine and D-glutamine could be measured in presence of abundant L-AAs, revealing concentrations of 1.1 ± 0.2 µM and 0.67 ± 0.23 µM, and enantiomeric ratios of 4.8%-8.0% and 0.34%-0.74%, respectively. The concentration and enantiomeric ratio for the glutamine enantiomers in CSF was similar as found in the study described in Chapter 2, but D-serine was not found in CSF with the CE-Flu method of Chapter 2.

6.2. Concluding remarks

Based on the data obtained and the practical experience gained, several aspects regarding the performance and applicability of CE-Flu and CE-MS using either the direct or the indirect separation approach are discussed below, and some general conclusions are drawn. Some key parameters of the four developed methods presented in this thesis are summarized in Table 1.

Table 1. Summary of the chiral CE methods for AAs developed in the thesis.

Chapter	Separation method	Derivatization agent	Detection	BGE	LOD (injected concentration; nM)	LOD (sample concentration; nM)	Number of enantioseparated AAs (Rs ≥ 1.2)
2	MEKC	FMOC	Flu	40 mM sodium tetraborate (pH 9.5) containing 15% isopropanol (v/v), 30mM SDS and 30 mM β-CD	14-98 (tryptophan, 536)	280-1960	15
3	CE	FMOC	MS	50 mM ammonium bicarbonate (pH 8) containing 15% isopropanol (v/v) and 10 mM β-CD	500-8300 (cysteine, 12500; tyrosine, 84300)	5200-84000	9
4	MEKC	FLEC	Flu	40 mM sodium tetraborate (pH 9.2) containing 15% isopropanol (v/v) and 25 mM SDS	13-60 (arginine, 156; tryptophan, 580)	150-600 (arginine, 1560; tryptophan, 5800)	12
5	MEKC	FLEC	MS	150 mM APFO (pH 9.5)	130-630	380-4620	14

6.2.1. Chiral CE-Flu

Derivatization

Flu detection for the chiral CE analysis of AAs has been performed in order to achieve sensitive detection of the D-forms, which often are at low concentrations in biological samples. In order to allow Flu detection, derivatization of the AAs with the fluorescent agents FMOC and FLEC was employed in, respectively, direct and indirect CE separation approaches. The analogous agents exhibit very similar excitation and emission properties and react with primary and secondary amines. The main advantage of FMOC and FLEC is their fast derivatization reaction (few min) compared to other common derivatization agents (hours up to overnight). FMOC and FLEC have been used before for chiral CE analysis of AAs, however, detection of the fluorescent derivatives was so far performed by UV-absorbance detection. This is most probably due to the fact that standard commercial Flu detectors for CE do not provide the UV light sources required for excitation of FMOC and FLEC. Interestingly, FMOC and FLEC not only add a fluorophore to the respective AAs, but also enhance the enantioseparation of DL-AAs. While derivatization of the AAs with a chiral agent like FLEC obviously is essential to allow separation of DL-AAs as their diastereomers in a non-chiral system (indirect approach), derivatization with FMOC also aided separation of AA enantiomers when using β-CD as chiral selector in the BGE. Actually, without FMOC derivatization no chiral separation of AAs was observed. Probably, inclusion in and interaction with the chiral selector becomes more discriminating when the AAs are derivatized [3].

Sensitivity

Using either the direct or indirect chiral separation approaches, similar LODs (injected AA concentration) were achieved for most of the proteinogenic DL-AA, ranging from 14 to 98 nM (direct) and 13-60 nM (indirect) (Table 1). With both approaches, DL-tryptophan showed less favorable LODs (536-580 nM), which is probably caused by intramolecular quenching of the FMOC and FLEC emission by the indole moiety of tryptophan.

In order to reduce the adverse effect of sample matrix components and non-reacted derivatization agent on the CE separation, the derivatized samples had to be diluted with water prior their analysis (a very common procedure in CE analysis of AAs). As a consequence, LODs expressed in actual sample concentrations, are 10-20 times higher, yielding values of 280-1960 nM for the direct approach (using FMOC) and 150-600 nM for the indirect approach (using FLEC). LODs for DL-AAs obtained in CSF were similar to the ones obtained for aqueous solutions, indicating minor effects

of the residual sample matrix on the AA analysis. Overall, the LODs achieved with Flu detection encompass a sensitivity improvement of up to three orders of magnitude for specific AAs as compared to chiral analysis of FMOC-and FLEC-DL-AAs by CE with UV absorbance detection. The achieved LODs obtained with the two developed CE-Flu methods are comparable to LODs obtained with chiral CE-Flu of AAs using other derivatization agents [4-7].

Separation performance

Both of the developed CE-Flu methods show good enantioresolution for most chiral proteinogenic AAs. While previous chiral CE-Flu works focused on few AAs only, one of the main goals of the presented studies was the optimization of the separation performance for multiple proteinogenic AAs. From the proteinogenic AAs, 15 DL-AAs and 12 DL-AAs were successfully enantioseparated (resolutions above 1.2) using the direct and the indirect separation approach, respectively (Table 1). In both CE-Flu methods, SDS was added to the BGE to enhance chemo- and enantioseparation by employing MEKC. However, strong affinity of doubly derivatized AAs for the micelles did not allow detection of cysteine, tyrosine and lysine, within 1-hour analysis time. The acidic and negatively charged AAs glutamic acid and aspartic acid, on the other hand, show electrostatic repulsion from the negatively charged micelles and therefore did not show enantioseparation. Probably due to the spatial conformation of its derivatives, proline could also not be enantioseparated. Overall, it seems that more AAs can be chirally separated when using the direct separation approach (with chiral selector in BGE). However, taking also the chemoseparation of the AAs into account, no significant difference in selectivity was observed (10 chiral AAs could be chirally analyzed simultaneously, in both methods, with resolution of 1.2 or higher in one run).

6.2.2. Chiral CE-MS

MS was introduced as detection technique for chiral CE of AAs in order to achieve unambiguous assignment of the AA enantiomers in the presence of matrix components and co-migrating AAs. Selectivity may also result in better signal-to-noise ratios for AAs and thus enhanced sensitivity. On the other hand, when using the direct separation approach, the chiral selector in the BGE, which co-migrates with the analytes into the ESI source, induces analyte ion suppression and reduction of MS signal intensities. Partial capillary filling or reverse-migrating phases previously have been used to avoid the chiral selector from entering the ESI source. However, with these methods, chiral separation often is compromised and AA-specific optimization is

needed. Direct coupling of chiral CE to MS offers unaltered chemo- and enantio-separation, but the effect of the chiral selector in the BGE has to be studied and optimized in order to achieve both satisfactory enantioseparation and modest analyte ion suppression. Alternatively, AAs can be transformed to diastereomers using a pure chiral reagent, which will enable the separation of the formed AA diastereomers without chiral selector in the BGE, and thus may allow higher sensitivity, although the chemo- and enantio-separation has to be investigated.

Sensitivity

In the direct chiral CE-MS method presented in this thesis using 10 mM β-CD in the BGE, LODs for most DL-AAs were in the 0.5-8.3 µM range. For the presented indirect chiral CE-MS method, where there was no chiral selector in the BGE, analyte ion suppression was significantly lower and LODs were in the range of 130-630 nM for the FLEC-derivatized DL-AAs (i.e. 10-times better sensitivity on average for most DL-AAs). Remarkably, the used BGE of AFPO showed less ion suppression than a BGE of ammonium acetate (one of the most commonly used BGEs in CE-MS).

For both developed CE-MS methods, the LODs achieved for DL-AAs in CSF were similar to LODs obtained for DL-AAs in aqueous solutions, indicating minor sample matrix effects on the AAs analysis. Using the direct CE-MS method, deproteinization of the CSF was performed before derivatization. For the indirect CE-MS method, the CSF sample preparation involved buffer adjustment and pH adjustment, being significantly simpler and faster than the former procedure, but also resulting in a more diluted sample. In both methods, the CSF samples were derivatized and diluted before injection (1:1, v/v, in order to reduce the organic solvent content in the sample), resulting in LODs of 5-84 µM and 380-3260 nM for most enantioseparated AAs using the direct or indirect method, respectively (Table 1). Again, it is clear that circumventing the chiral selector in the BGE and using APFO as BGE, enhances the MS signal-to-noise ratios, gaining about an order of magnitude for the analysis of chiral AAs by CE-MS.

As was shown for the CE-Flu methods, the derivatization of DL-AAs with FMOC and FLEC has been crucial for achieving enantioseparation with both of the developed CE-MS methods, either because enantio-discriminative interactions were enhanced, or because of the formation of separable FLEC-AA diastereomers was achieved. LODs were at the µM range for the direct CE-MS approach and at the nM range for the indirect CE-MS approach, and are overall comparable to the LODs obtained by methods using other derivatization agents.

Separation performance

With the direct CE-MS method using β-CD, all the chiral proteinogenic AAs could be detected with fifteen AAs also enantioseparated with a resolution of 0.5 or higher. Enantioresolutions of 1.2 and higher were achieved for nine AAs. A few AAs were detected, but not enantioseparated, maybe because of their too small residual group that may reduce their specific chiral interaction with the chiral selector, limiting enantiomeric discrimination (alanine), or their overall positive charge led to fast migration towards the capillary outlet leaving insufficient time for enantioseparation (arginine). The lower number of enantioseparated AAs obtained with the direct CE-MS method than with the direct CE-Flu method (nine vs. sixteen; Table 1) is probably due to the extra selectivity provided by MEKC in the latter method. Indeed, the direct CE-Flu method employing SDS in the BGE yielded higher chemo- and enantioseparation of the DL-AAs. Use of SDS in the direct CE-MS method is, however, not possible as it will cause a severe analyte signal suppression. When using the indirect chiral CE-MS method, sixteen chiral AAs could be detected, with most of them enantioseparated with a resolution of 1.2 or higher. Some AAs were not detected, probably because of their high affinity to the micelles (tyrosine and cysteine) that led to very slow migration. Some other AAs were detected, but exhibited a poor peak shape. This was the case for proline, which has a secondary amine functionality that probably affects the spatial orientation of the FLEC moiety and thus the diastereomer separation. AAs with double negative charge, as aspartic acid and glutamic acid, are prevented from partitioning in the negatively charged micelles and therefore are not enantioseparated. Interestingly, the detectability and enantioseparability of most AAs was similar as obtained with the indirect CE-Flu method, with the exception of histidine and lysine. While these two AAs were not detected by the indirect CE-Flu method, due to their high affinity to the SDS micelles (migration times above 60 min), they were detected and enantioseparated in a reasonable time by the indirect CE-MS method, suggesting a lower affinity of these DL-AAs to the APFO micelles than to the SDS micelles. Thus, the indirect chiral CE-MS method enables the analysis of 14 DL-AAs in a single run with satisfactory resolutions (≥ 1.2).

Main reason for the lower number of enantioseparated DL-AAs obtained in the direct CE-MS method than in the indirect CE-MS method (Table 1) is that the chemo- and enantioseparation in the latter method were enhanced by the present pseudostationary phase. The chemoseparation in the direct CE-MS method is largely based on differences in charge-to-size among the AAs, and the enantioseparation on the specific chiral recognition by the β-CD chiral selector towards the enantiomers. The resulting lower chemoselectivity and a smaller separation time window than

achieved with MEKC, probably affects the overall selectivity of the direct CE-MS method.

6.2.3. Applicability and perspectives

The direct CE-Flu method was applied to the analysis of CSF, in which endogenous levels of D-aspartic acid and D-glutamine could be detected at enantiomeric ratios of 19.6% and 0.35%, respectively, showing the ability to detect down to 300-times lower levels of D-AAs next to abundant L-AAs. This may allow the determination of enantiomeric ratio changes of AAs in CSF, which are relevant in fields such as early clinical diagnostic [8-10].

The dilution of the derivatized sample before injection, which is needed to avoid interferences by matrix components and overloading of the unreacted FMOC, may be circumvented by achieving improved sample derivatization and cleanup. As Campíns-Falcó et al. [11] indicated for the derivatization of several AAs with FMOC, efficient derivatization and sample cleanup could be achieved using SPE. In this procedure, AAs containing solutions were loaded on C18 SPE cartridges, followed by injection of an FMOC-solution. After 2-min incubation time allowing the C18-adsorbed AAs to react with the FMOC, the derivatized analytes were eluted using acetonitrile-water (1:1, v/v). This resulted in an extract with less interfering matrix components and less unreacted FMOC in sample preparation times not longer than used in this thesis. Such sample cleanup procedure might allow the detection of D-AAs at the intrinsic sensitivity of the CE-Flu system (i.e. down to the low nanomolar range).

The high detection selectivity that both developed chiral CE-MS methods show, enables the enantioseparation of up to 14 chiral proteinogenic DL-AAs in CSF with resolutions of at least 1.2 in a single run. This is an improvement with respect to previously reported chiral CE-MS works, which mostly focus on either non-proteinogenic AAs (such as ornithine and carnitine) or only few proteinogenic AAs. Furthermore, as for the CE-Flu methods, the use of FMOC and FLEC as derivatization agent in the CE-MS methods resulted in simpler and shorter sample preparation times with respect to most previously described chiral CE-MS works where other derivatization agents were used (with up to an overnight reaction time), and consequently, the overall analysis time when using FMOC and FLEC was significantly shorter. The new indirect chiral MEKC-MS approach employing a volatile surfactant seems to be more attractive, as more DL-AAs could be enantioseparated, at higher chemo- and enantioresolution, and with better LODs, enabling e.g. the detection of endogenous D-serine and D-glutamine in CSF. Even though the direct CE-MS method

showed adequate performance regarding the number of enantioseparated AAs and their LODs, endogenous D-AAs in CSF were not detected.

Further sensitivity improvement might be achieved by applying on-line electrophoretic pre-concentration approaches (e.g. pH-mediated stacking), by using SPE as described above [11], or by avoiding sample dilution. For example, with both CE-MS methods the derivatized samples are diluted with water in order to reduce the percentage of organic solvent in the sample solution. Drying of the sample and reconstituting it in a solution with lower organic solvent content may not only spare the need of such dilution, but can also increase the sample concentration. Sensitivity enhancement in MS detection could be achieved by using sheathless CE-MS interfacing instead of SL-based coupling. This will circumvent dilution of the CE capillary effluent by the SL, and may result in up to 50-fold lower detection limits [12]. These optional procedures may bring the LODs to the low nM range, enabling the detection of more D-AAs in biological samples. Lastly, in the light of the results obtained for the indirect CE-MS method, it would be interesting to examine the effect on selectivity and sensitivity by using APFO in the BGE of the direct CE-MS method. MEKC may enhance the chemo- and enantioselectivity in direct CE separation, as demonstrated for CE-Flu method. This may even allow to reduce the chiral selector concentration in the BGE, thus improving the method sensitivity.

Other separation techniques enabling the analysis of AA enantiomers in complex samples, such as LC and GC, have been used more frequently than CE. However, as compared to CE, these techniques may show limited efficiency and peak capacity in chiral separation, [13]. In addition, as discussed by Szoko et al. [13], although both direct and indirect chiral separation approaches are applicable in LC, GC and CE analysis, there often is a preferred mode: chiral stationary phases (CSPs) for LC usually possess poor chemical selectivity, and thus often need an additional selectivity as achieved by e.g. 2D-LC or LC-MS. Therefore, chiral LC often is performed by the indirect approach, using conventional reversed-phase (RP) columns. For chiral GC, the analysis of D-AAs often is performed by using the direct approach, employing CSPs (e.g. Chirasil-L-Val). As for chiral CE, although the direct approach has been used almost exclusively in AA analysis, the methods presented in this thesis show that highly efficient chiral CE analysis can be achieved using both separation strategies. Combining the CE separation with the presented detection techniques provided further sensitive and selective approaches for chiral AA bioanalysis.

Using LC-based methods for the analysis of chiral AAs in biological matrices [13-16], LODs were in the ranges of 15-800 nM, 1-150 nM and 0.1-1000 nM using UV absorbance, Flu and MS detection, respectively. For GC-based methods, LODs in the

ranges of 0.3-40 nM and 2.5-3200 nM were reported using flame-ionization and MS-detection, respectively. Those concentration values are somewhat better than the LOD concentrations achieved by the CE methods reported in this thesis (150-2000 nM and 380-84000 nM for the CE-Flu and CE-MS, respectively). However, considering the low CE injection volumes, compared to the injected volumes in those LC and GC methods (about 10-35 nL vs. 5-10 µL and 0.5-1 µL, respectively), the absolute analyte amount LODs of the CE methods in this thesis are favorable. Furthermore, one of the main advantages of the CE methods presented in the thesis is their relative simplicity. All methods were performed on common bare fused-silica capillary without the need of coatings or long regenerations between runs. Also, the commercially available native β-CD was used in the direct chiral separation methods. The use of FMOC and FLEC as fast reacting derivatization agents, the short sample pretreatment prior the analysis, and the simply to prepare BGEs, added to the overall simplicity and cost-effectiveness of the developed methods.

Integration of the AA derivatization step into the CE procedure would be an interesting direction for further study. In-capillary derivatization has been described for a variety of analytes and its reported advantages include low consumption of reagents and samples, limited sample dilution, and the possibility of automation. Derivatization in the capillary would simplify the sample preparation procedure, avoiding manual steps and transfer of sample. The feasibility of in-line derivatization of DL-AAs with FMOC and FLEC has been demonstrated in CE-UV and CE-MS settings [17-20]. Clever mixing schemes by injection of sample and reagent plugs and voltage programming, provided in-line AA derivatization directly followed by enantioseparation. Considering the LODs obtained so far for DL-AAs (ranging from the low µM to the mM range), improvement of detection sensitivity seems indicated. In that respect, the incorporation of the Flu detection described in this thesis in an in-capillary setup may be a fruitful option. Moreover, for the analysis of real samples, optimization of the in-capillary derivatization and chiral separation is still needed, in particular in combination with MS, which seems the most proper detection method for complex samples.

Whereas the CE-Flu methods are intrinsically highly sensitive and enable the detection of D-AAs at the low nM level, the CE-MS methods show higher selectivity and therefore are more appropriate for the analysis of a large number of AAs in complex samples in a single run (Table 1). Using the two detection principles within one CE setup (i.e. CE-Flu-MS) seems attractive and has actually been demonstrated for e.g. glycan analysis [21, 22]. Combination of the two detection approaches described in the thesis looks feasible. The Flu detection cell could be positioned on the capillary

at 15-20 cm from the capillary outlet, from where the effluent can be sprayed into the ion source of a mass spectrometer. Successful implementation of such CE-Flu-MS could provide both high selectivity and sensitivity and might allow the analysis of D-AAs in complex samples at low levels.

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